AD NUMBER

AD831372

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BDRL D/A ltr, 29 Sep 1971

AD

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EFFECT OF BACTERIAL CELL MOISTURE ON THE SPORICIDAL ACTIVITY OF BETA-PROPIOLACTONE VAPOR

Robert K. Hoffman

MARCH 1968

DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

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EFFECT OF BACTERIAL CELL MOISTURE ON THE SPORICIDAL ACTIVITY OF BETA-PROPIOLACTONE VAPOR

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Project 1B622401A072

March 1968

ACKNOWLEDGMENTS

The author acknowledges the technical assistance of George L. Gilbert and Mrs. Beatrice C. Rowe.

ABSTRACT

The atmospheric relative humidity is one of the factors that is generally stated as affecting the activity of a vapor phase disinfectant. Data are presented to show that, in beta-propiolactone (BPL) vapor disinfection, the important factor is, in reality, the moisture content and location of water in the cell and not necessarily the atmospheric RH. Previous studies revealed that only about 50% of the bacterial spores equilibrated to 45% RH were killed when exposed at the same RH to BPL vapor. On the other hand, all the spores equilibrated to and then exposed at 75% RH to BPL were readily killed. Studies presented in this paper show that spores equilibrated to 98% RH are readily killed by BPL at 45% RH, but only 99% of the spores equilibrated to 75% RH are killed by BPL at 45% RH. Data also show that, to be killed, desiccated spores must be exposed to BPL at higher humidities than would be required if the spores had not been desiccated.

I. INTRODUCTION

Beta-propiolactone (BPL) was shown by Hoffman and Warshowsky[1] to be an effective vapor phase disinfectant; however, its activity was very dependent on the atmospheric humidity. For example, the chemical showed great activity at relative humidities (RH) of 75 and 85%, considerable activity at 60% RH, and little activity at 45% RH. No data were presented for RH's above 85%. We suspected that, in reality, it is the amount of moisture as well as its location within the cell that are the significant factors regulating the rate BPL kills the cell and not necessarily the amount of water in the atmosphere surrounding the cell. As shown by Bateman et al.[2] and Gilbert et al.,[3] the amount of moisture in a bacterial cell depends not only on the relative humidity of the atmosphere surrounding the cell but also on the past history of the cell; i.e. whether or not the cell was previously subjected to desiccation. Their studies revealed that cells have less moisture when equilibrated from a highly desiccated state to a higher RH, except at saturation RH, than do cells that have been equilibrated to the same RH starting from the wet state. A similar hysteresis effect was shown by Katchman and McLaren in 1951[4] for tobacco mosaic virus. As far back as 1936, Speakman and Cooper[5] reported the same type hysteresis effect with wool.

The work of Gilbert et al.[3] tended to dispell the hypothesis that it is only the amount of cellular water that regulates the rate of microbial inactivation by ethylene oxide. Their results led us to suspect that it is also the location of the water in the cell that is involved.

This paper was written for the express purpose of presenting some observations made in our laboratories on the effect bacterial cell moisture content has on the rate cells are inactivated by BPL.

II. MATERIALS AND METHODS

A. PREPARATION OF TEST MICROORGANISMS AND SAMPLES

The effect of cell moisture content on the activity of BPL vapor was shown by the rate that bacterial spores preconditioned to humidity are killed when exposed to the chemical vapor at various RH measurements.

The test organisms were Bacillus subtilis var. niger spores. They were grown in liquid casein acid digest media, harvested by centrifugation, washed, suspended in water and heat shocked at 60 C for 30 minutes to kill the less resistant vegetative cells. Small patches of cotton or

Whatman No. 42 filter paper were contaminated with 0.1 ml of B. subtilis var. niger spore suspension adjusted to give a final concentration of 1 to 10 million viable spores per patch. The patches were then transferred to desiccators charged with the various saturated salt solutions shown below to give the desired relative humidity for preconditioning at 25 C.

Saturated Salt Solution	RH, %
Potassium Dichromate	98
Potassium Chloride	85
Sodium Chloride	75
Nickel Chloride	53

Anhydrous calcium sulfate was used in another desiccator to give a relative humidity of less than 1%. The contaminated patches were preconditioned to the desired RH for approximately one week prior to use for test purposes.

B. EXPOSURE TO BPL VAPOR

The data presented here were collected in two separate experiments. The first (Experiment A) was carried out in a modified 10-liter desiccator, whereas the other was carried out in a large chamber in which both the temperature and RH could be controlled (Experiment B).

In Experiment A, the preconditioned contaminated patches were placed in the modified desiccator and the RH therein adjusted rapidly to the desired level by flushing with air of that RH. The RH of the air was regulated by blending the correct proportions of two air streams, one saturated by passing through water and the other dehydrated by passing through a drying column containing calcium sulfate. A wet-dry bulb apparatus attached to the downward side of the modified desiccator was used to determine when the desired RH was attained. Once the desired RH was achieved, a slight but predetermined negative pressure was drawn in the chamber, and then the pressure was returned to atmospheric by bleeding in BPL vapor. The use of such a chamber permits only one BPL exposure time per test because it is necessary to remove the top of the desiccator to retrieve the patches. Once opened, the internal conditions are completely altered. This technique requires numerous such tests in order to establish a death rate. Periodically, the air in the desiccator was checked for BPL content just before opening to retrieve the patch for assay.

Experiment B was conducted in a 1,700-liter chamber. In this chamber, the temperature, RH and BPL concentrations were first adjusted, then a long rod was inserted with a number of spore-contaminated patches attached by pins. Periodically, the rod was withdrawn only far enough to permit removal of several patches for assay. In this way, a complete death rate study was conducted in one chamber experiment.

Both the control patches (inoculated with the microorganism but not exposed to BPL) and the exposed patches were assayed by immersion in dilution blanks containing 0.1% sodium thiosulfate (to neutralize any BPL carried over on the patch) and 0.05% Tween 20 (to aid the removal of organisms from the patch). Each dilution blank was shaken vigorously, and aliquots were plated in nutrient agar. All plates were incubated 48 hours at 37 C before counting. Duplicate patches were used for each test point, and each test was repeated three times.

The following protocols were used:

Experiment A: Microorganisms were preconditioned on patches to 1% RH, and death rates were determined upon exposure to BPL at 75, 85, or 92% RH.

Experiment B: Microorganisms were preconditioned on patches to 98, 75 and 53% RH, and death rates were determined upon exposure to BPL vapor at 45% RH.

III. RESULTS AND DISCUSSION

The results shown in Figures 1 through 3 indicate that the moisture content of the microorganism is of major importance in regulating the rate the spores are inactivated by BPL. Figure 1 shows that only 40 to 50% of the spores preconditioned to 45% RH and then exposed to BPL at the same RH are killed by the chemical vapor. Yet, as seen in Figure 2, all of the spores were killed by BPL at 45% RH if they were first preconditioned at 98% RH. Even so, the rate the cell loses moisture at a lower RH is quite rapid because the cells preconditioned at 75% were killed fairly rapidly at first and then with diminished rapidity until there was no further kill after a 99% reduction. This contrasts with the rapid and complete kill of spores equilibrated at 75% RH and then exposed to BPL at 75% RH (Fig. 1).

The importance of cell moisture in BPL sterilization is further emphasized by the results shown in Figure 3. Here, it is seen that a small percentage of spores preconditioned at 1% RH are thereafter very resistant to BPL sterilization at 75% RH. This is the same effect that was noted with ethylene oxide;[3] in that case, however, the resistance to sterilization by ethylene oxide is overcome only by first subjecting the cells to drastic measures, such as physically wetting with water or equilibrating the organisms for 4 and 6 days at 98 and 75% RH, respectively. With BPL on the other hand, it is much easier to overcome the resistance: merely exposing the cells to BPL at about 92% RH is sufficient; no wetting or previous lengthy preconditioning to a high RH is required.

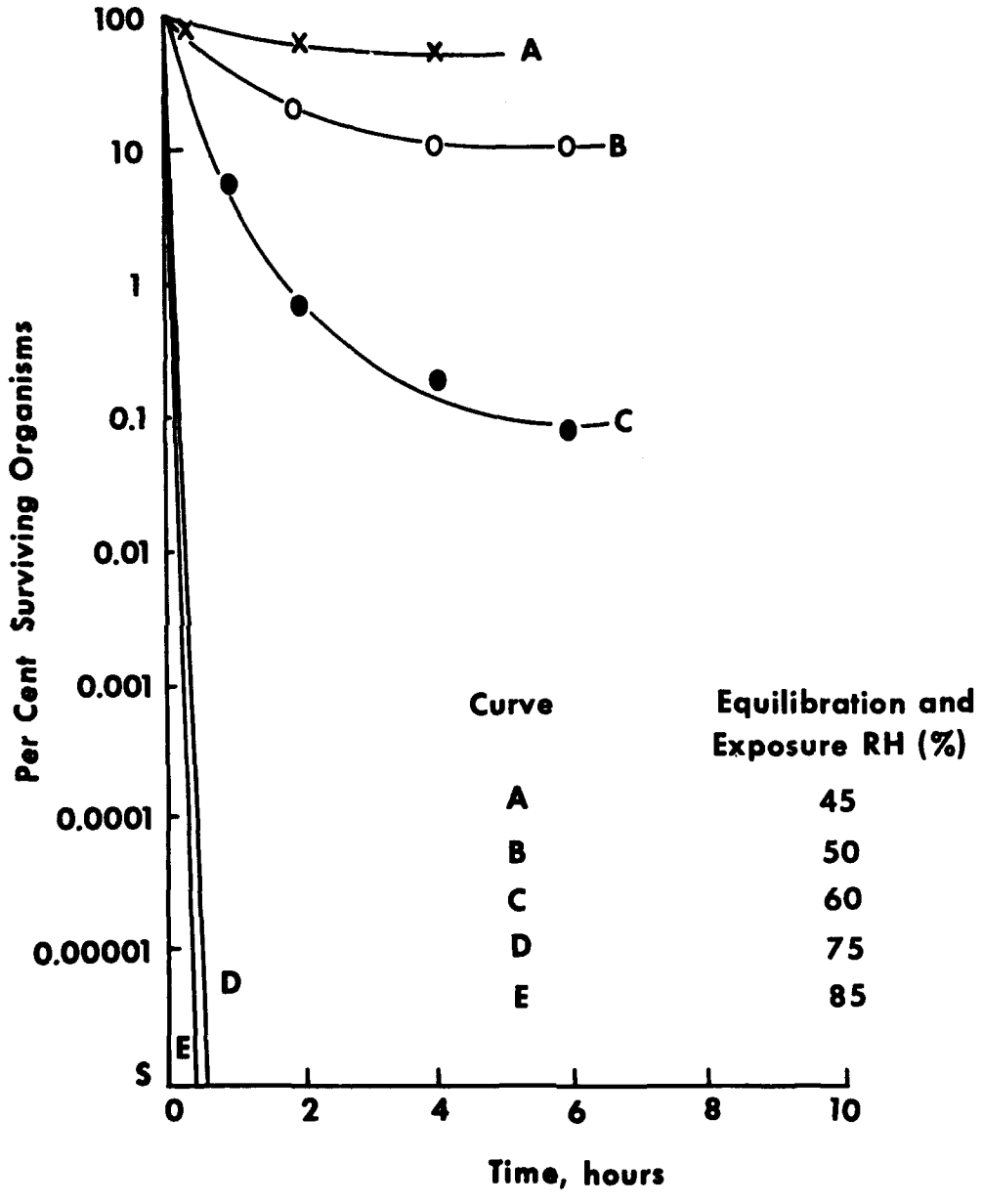

Figure 1. Effect of Relative Humidity on Death Rate of B. subtilis var. niger Spores Exposed to 1.5 mg BPL/liter, 27 C, and Various Relative Humidities (from Hoffman and Warshowsky[ ]).

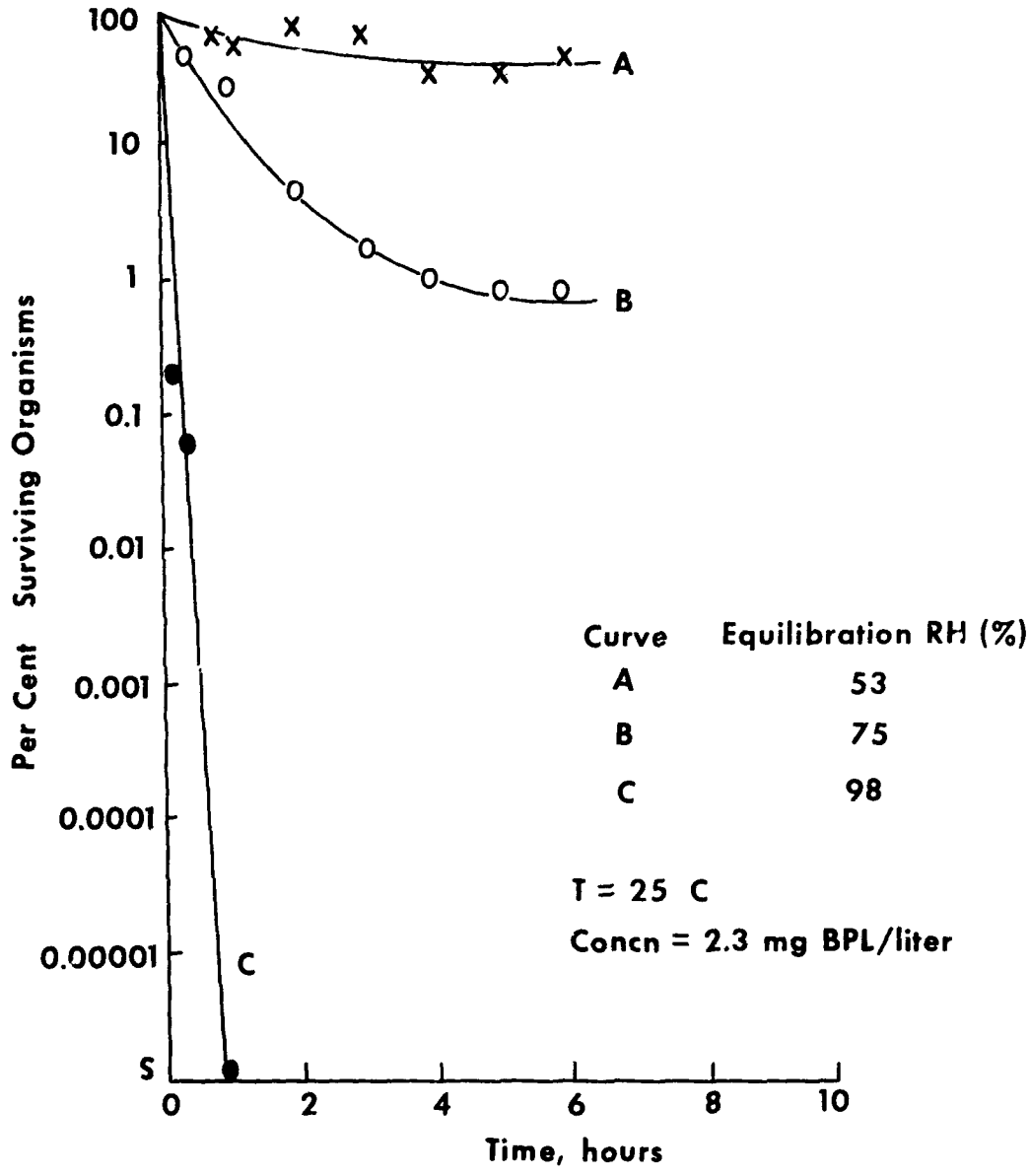

Figure 2. Rate of Kill of B. subtilis var. niger Spores Equilibrated to 98, 75, 53% RH and Exposed to BPL Vapor at 45% RH.

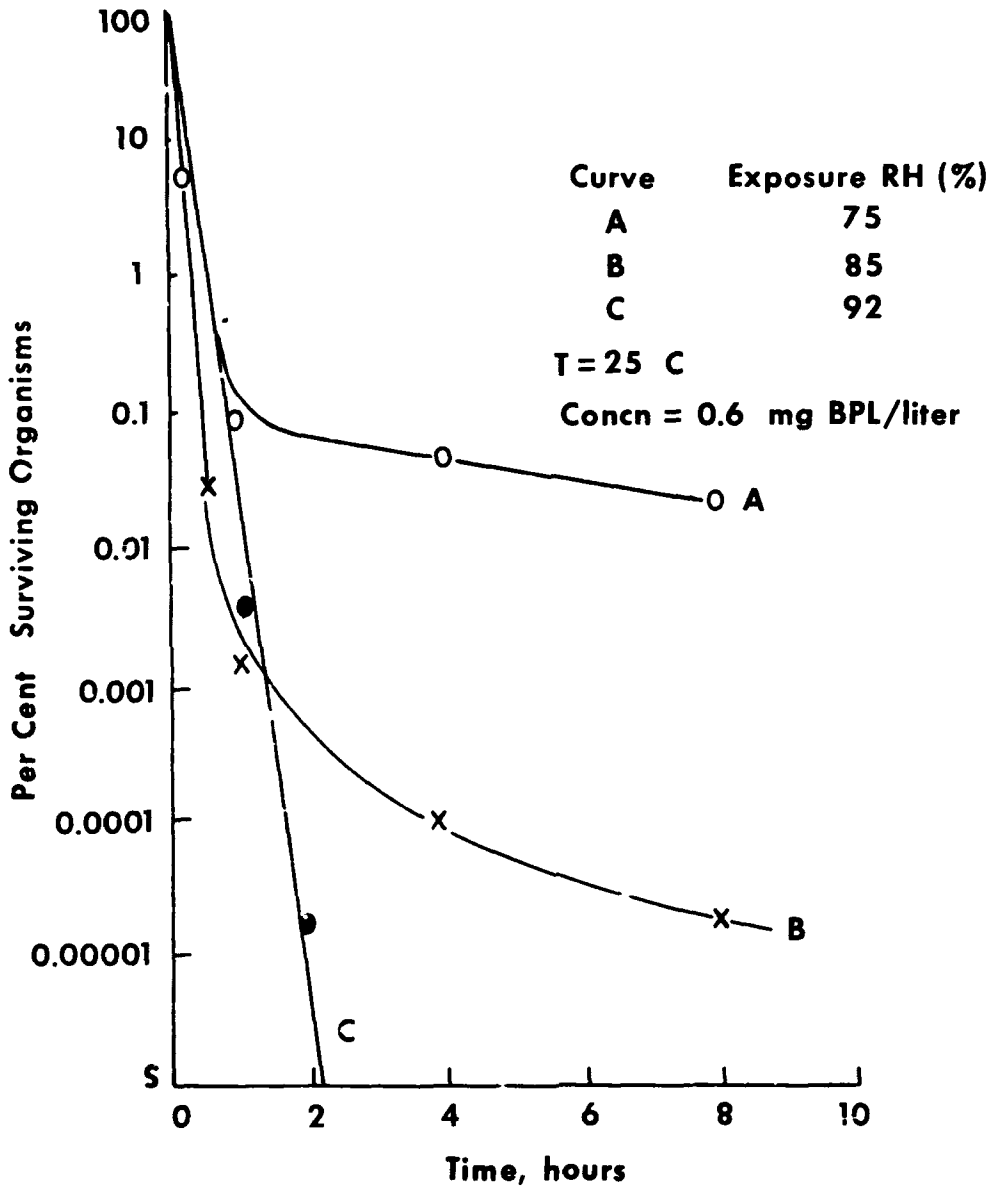

Figure 3. Rate of Kill of B. subtilis var. niger Spores Equilibrated to 1% RH and Exposed to BPL Vapor at 92, 85, and 75% RH.

Gilbert et al. showed (Fig. 4) that B. subtilis spores equilibrated at 25 C from a wet spore suspension to 75% RH contain about 23% moisture, whereas cells that have been desiccated and then conditioned to 75% RH contain only about 19% moisture. The cell on the adsorption curve must now be equilibrated to about 85% RH to contain 23% moisture. This relative humidity approaches but does not quite meet that needed to overcome the resistance to BPL sterilization after the cell was subjected to desiccation (Fig. 3). This fact would make it appear that, although the amount of moisture present in the cell is of prime importance, its location is also of extreme importance in regulating the rate it is killed by BPL.

The curves shown in Figure 1 were obtained with bacterial spores equilibrated from a wet suspension. Therefore, the cells equilibrated to 60% RH, according to Figure 4, would contain about 18% moisture. Curve A, Figure 3, was obtained at 75% RH but closely resembles the 60% RH curve (C) in Figure 1. Actually, the spores used to obtain the A curve of Figure 3 were tested at 75% RH, but this was after the cells were desiccated. Such cells, when in equilibrium at 75% RH, also should contain 18 to 19% water. Thus, the cell moisture content will be the same in both cases, and the curves resemble each other, but only if the rate of moisture transfer into the cell is quite rapid. The similarity of the curves verifies the rapid moisture transfer into the cell.

The role of moisture in BPL cell inactivation is not clear. Furthermore, it is even more difficult to explain in the light of experience with ethylene oxide, a chemical that presumably inactivates by the same mechanism as BPL but requires little water to kill the cell. As shown by Kaye and Phillips,[6] ethylene oxide is most active at about 30% RH; however, even at much lower RH's, ethylene oxide will kill 99.9% of the cells thus exposed.[3] BPL is a very reactive chemical and should require no more water than does ethylene oxide to alkylate the various protein groups; yet, an RH of 70% or higher is needed for greatest BPL activity. Black and Gerhardt[7] proposed the hypothesis that the dormant spore has an outer fully permeable coat, a cortex that is possibly lipid-like, and a dense core that is an insoluble and heat-stabile gel. The core is believed to be permeable only to small molecules, but the cortex is believed to be permeable to water and small lipophilic solutes. Possibly, the reason that BPL requires a high cell moisture content to inactivate is that water, or some cell constituent solubilized by it, acts as a transport agent. The possible lipid-like nature of the cortex and the density of the core could conceivably require more moisture for the transport of the slightly larger and less lipid-soluble BPL molecule to the site or sites of reaction than is required for ethylene oxide. If this is the case, it would further indicate that the location of the water in the cell is of extreme importance.

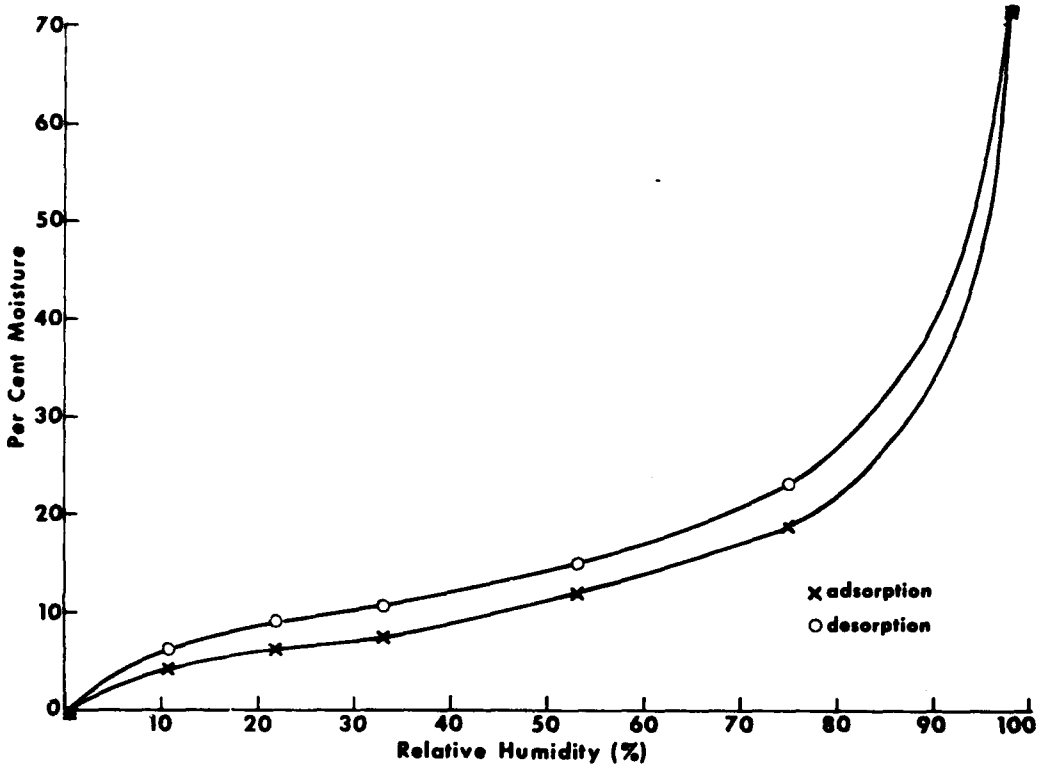

Figure 4. Moisture Content of B. subtilis Spores as a Function of Relative Humidity at 25 C.

The mechanism by which BPL inactivates the bacterial cell has not been definitely established. BPL is a very reactive chemical and can alkylate many terminal groups found on many protein molecules, such as amino, imino, hydroxyl, and carboxyl radicals (Jones and Lundgren[8]). Searle[9] showed that BPL reacts with thiol and disulfide groups in albumin. Ichikawa et al.[10] concluded from their studies that BPL will react with sulfhydryl groups of enzyme proteins as well as the N_7 position of the RNA or DNA guanine moiety. Roberts and Warwick[11] previously suggested the latter as the site of BPL reaction. More recent studies with other alkylating agents, such as ethylene oxide and propylene oxide, also implicate the N_7 guanine position as the main site of reaction in RNA or DNA. It remains to be determined, however, whether a microorganism is inactivated by one or several alkylations of a vital site in the cell or whether it requires a number of alkylations of amino, sulfhydryl, hydroxyl, and/or carboxyl protein radicals, thus generally poisoning the cell. Whatever the lethal reaction between BPL and the microbial cell, it obviously cannot proceed without considerable water in the right location.

LITERATURE CITED

1. Hoffman, R.K.; Warshowsky, B. 1958. Beta-propiolactone vapor as a disinfectant. Appl. Microbiol. 6:358-362.

2. Bateman, J.B.; Stevens, C.L.; Mercer, W.B.; Carstensen, E.L. 1962. Relative humidity and the killing of bacteria; the variation of cellular water content with external relative humidity or osmolality. J. Gen. Microbiol. 29:207-219.

3. Gilbert, G.L.; Gambill, V.M.; Spiner, D.R.; Hoffman, R.K.; Phillips, C.R. 1964. Effect of moisture on ethylene oxide sterilization. Appl. Microbiol. 12:496-503.

4. Katchman, B.; McLaren, A.D. 1951. Sorption of water vapor by proteins and polymers: IV. J. Amer. Chem. Soc. 73:2124-2127.

5. Speakman, J.B.; Cooper, C.A. 1936. The adsorption of water by wool: I. Adsorption hysteresis. J. Textile Inst. 27:183T-196T.

6. Kaye, S.; Phillips, C.R. 1949. The sterilizing action of gaseous ethylene oxide: IV. The effect of moisutre. Amer. J. Hyg. 50:296-306.

7. Black, S.H.; Gerhardt, P. 1962. Permeability of bacterial spores: IV. Water content, uptake and distribution. J. Bacteriol. 83:960-967.

8. Jones, H.W.; Lundgren, H.P. 1950. Modification of wool with beta-propiolactone. U.S. Patent 2,517,573.

9. Searle, C.E. 1961. Polarographic experiments on the interaction of β-propiolactone with albumin thiol and disulphide groups. Biochim. Biophys. Acta 52:579-582.

10. Ichikawa, Y.; Utsumi, S.; Kurisu, K. 1967. Studies on the antimicrobial action of propionate and related substances: I. The mechanism of the microbial action of β-propiolactone. Kyoto Univ. Ind. Arts Textile Fibers 41:84-93.

11. Roberts, J.J.; Warwick, G.P. 1963. The reaction of β-propiolactone with guanine, desoxyguanylic acid and RNA. Biochem. Pharm. 12:1441-1442.

DOCUMENT CONTROL DATA - R & D

(Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified)

1. ORIGINATING ACTIVITY *(Corporate author)*
Department of the Army
Fort Detrick, Frederick, Maryland, 21701

2a. REPORT SECURITY CLASSIFICATION
Unclassified

2b. GROUP

3. REPORT TITLE
EFFECT OF BACTERIAL CELL MOISTURE ON THE SPORICIDAL ACTIVITY OF BETA-PROPIOLACTONE VAPOR

4. DESCRIPTIVE NOTES *(Type of report and inclusive dates)*

5. AUTHOR(S) *(First name, middle initial, last name)*
Robert K. Hoffman

6. REPORT DATE	7a. TOTAL NO. OF PAGES	7b. NO. OF REFS
March 1968	15	11

8a. CONTRACT OR GRANT NO.	9a. ORIGINATOR'S REPORT NUMBER(S)
b. PROJECT NO. 1B622401A072	Technical Manuscript 442
c.	9b. OTHER REPORT NO(S) *(Any other numbers that may be assigned this report)*
d.	

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11. SUPPLEMENTARY NOTES	12. SPONSORING MILITARY ACTIVITY
	Department of the Army Fort Detrick, Frederick, Maryland, 21701

13. ABSTRACT

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14. Key Words

Beta-propiolactone
*Decontamination
Relative humidity
*Vapors
*Moisture
*Cells